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Cold Agglutinin Antibody in a Hyperimmune Erythrocyte Antiserum (41469)

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Abstract. To examine whether an innocuous, nonbacterial-associated immunogen could generate a cold agglutinin autoreactive antibody response, rabbits were hyperimmunized with sheep erythrocytes. Of five animals immunized, one developed an autoreactive antibody population which agglutinated autologous and allogenic erythrocytes at temperatures below 37°. The cold agglutinin antibody activity was present in the IgM class immunoglobulin fraction since only the excluded volume of a Sephacryl S-200 serum fractionation, and purified IgM, contained detectable activity. The cold agglutinin antibody was hemolytic in the presence of guinea pig complement, since whole serum, and purified IgM, sensitized rabbit erythrocytes for lysis in a biphasic temperature hemolysis assay. In hemolytic inhibition assays, the cold agglutinin antibody was sugar specific. The relative sugar ligand specificity in this assay was shown to be *N*-acetylgalactosamine > melibiose > galactose > lactose. The hyperimmune anti-sheep erythrocyte serum agglutinated both sheep erythrocytes and Group C streptococcal vaccine at 4° and at 37°. These data suggest certain animals can respond to nonbacterial-associated immunogen with antibody specific for the immunogen, but which may cross-react with autologous sugar determinants.

Cold agglutinin (autoreactive erythrocyte) antibodies have been shown to be associated with infectious illness and other human disease (1–5), and may appear spontaneously in certain strains of mice (6). Also, cold agglutinin antibodies may appear during hyperimmunization of rabbits with certain bacterial vaccines (7–9). Because of these associations, it has been of interest to determine whether these particular autoantibodies arise in response to the infectious or immunizing agent or whether they arise nonspecifically as one result of general immune stimulation.

A recent investigation by our laboratory revealed a cold agglutinin antibody response in rabbits hyperimmunized with Group C streptococcal vaccine (10). The Group C streptococcal cell wall carbohydrate structure carries determinants identical to those present on the glycan portion of the Forssman glycolipid of sheep erythrocytes. These determinants are α-anomerically linked *N*-acetylgalactosamine disaccharide residues. These same residues are the immunodominant structures present on Group C carbohydrate (11). Both IgM and IgG cold agglutinin antibodies isolated from Group C streptococcal antisera reacted with *N*-acetylgalactosamine and Group C carbohydrate. These data therefore suggested that these particular cold agglutinin antibodies were induced in response to the Group C streptococcal cell wall carbohydrate.

The present investigation was initiated to examine whether a bacterial vaccine was necessary to induce the cold agglutinin response, or whether a similar antibody response might be generated by hyperimmunization of rabbits with sheep erythrocytes.

Materials and Methods. *Vaccine preparation and immunization protocol.* Preparation of vaccine was according to Lancefield (12). Briefly, bacteria were cultured overnight in Todd–Hewitt broth and vaccine prepared from washed, pepsin-digested cells. The procedure used for immunization was according to Herd and Spragg (13). Rabbits were immunized three

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times per week for 4–5 weeks, rested 3 months, and again immunized.

Sheep erythrocyte immunization protocol. Sheep erythrocytes in modified Alsever's solution were washed three times in 0.15 *M* NaCl and pelleted by centrifugation at 800 g. Red blood cells were resuspended in 0.15 *M* NaCl to 10% (v/v) and 1.0 ml injected iv three times per week for 5 weeks into five animals. Rabbits were bled from the central ear artery each week.

Hemagglutination assay. Allogenic rabbit erythrocytes were collected in Alsever's solution and washed in 0.02 *M* phosphate, 0.15 *M* NaCl buffer, pH 7.2, made 1% (w/v) in bovine serum albumin. Cells were resuspended in this buffer to 0.5% (v/v) and distributed in "v" type microtiter plate wells. The wells contained an equal volume of twofold-diluted whole or Sephacryl S-200 fractionated sheep erythrocyte antisera. Microtiter trays were stored at 4° overnight and the reciprocal of the highest dilution which yielded positive agglutination was recorded as the cold agglutinin antibody titer.

Biphasic temperature hemolytic assay. This assay has previously been described in detail (10). Essentially, a pretitrated excess amount of guinea pig complement was added to a 0.25% (v/v) suspension of rabbit erythrocytes in the presence of twofold serially diluted whole or fractionated antisera. After incubation at 4° for 60 min and 37° for 60 min the amount of hemolysis was determined by supernatant volume absorbance measurements at 413 nm. Inhibition in this assay was performed using a pretitrated amount of cold agglutinin antibody sufficient to yield 50% hemolysis under conditions described above. Preincubation of increasing amounts of varying inhibitors with this particular amount of antibody was performed for 3 hr at 4° prior to assay. Inhibition was calculated relative to an uninhibited control.

Polysaccharide preparations. Group C streptococcal carbohydrate was purified according to an acidified sodium nitrite procedure provided by E. Gotchlisch, The Rockefeller University, New York (personal communication). Type III pneumococcal polysaccharide was purified according to Campbell and Pappenheimer (14). Type XIV pneumococcal polysaccharide was the generous gift of Eli Lilly and Company, Indianapolis, Indiana.

Results. Rabbit erythrocyte agglutinating activity at 4° or 37° was absent from preinoculation sera, while at these temperatures the same sera agglutinated sheep erythrocytes to a 1:4 dilution. After immunization, all rabbits produced sheep red blood cell-reactive antibodies (reactive at both 37° and 4°). Only one rabbit, however, produced antibodies reactive against rabbit erythrocytes; and, reactivity was detectable only at 4°. For this particular rabbit's antiserum, the sheep erythrocyte hemagglutination titer increased from 1:4 to 1:1024 during the fifth week of immunization. In contrast, hemagglutination reactivity of whole serum with rabbit erythrocytes occurred during the first, second, and third weeks only, and was relatively weak (highest titer $< 1:32$), and occurred only at 4°. Reactivity with both human adult O-positive and cord blood erythrocytes occurred in the warm (titer $< 1:4$) and increased in the cold (titer $< 1:8$). None of the remaining animals' sera reacted with human or rabbit erythrocytes when tested at either 37° or 4°. Hemolytic activity toward rabbit erythrocytes in the biphasic temperature hemolytic assay reached, however, a titer of 1:256 during the third week of immunization. Since most cold agglutinin antibodies are of the IgM class, the anti-sheep red cell antiserum was fractionated into 19 S (excluded) and 7 S (included) components by Sephacryl S-200 chromatography and examined for erythrocyte autoreactivity.

Figure 1 shows the results of hemolytic assays performed upon individual Sephacryl S-200 fractions. As shown, it is clear that all of the cold agglutinin activity was confined to the excluded column fraction; these results in addition to Ouchterlony analysis with goat anti-rabbit μ chain antiserum and polyvalent goat anti-rabbit Ig antisera, showed that IgM was the only immunoglobulin component in these fractions. That the cold agglutinin activity was

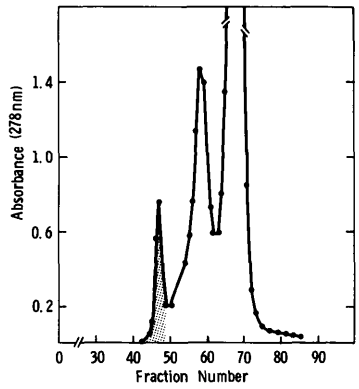

FIG. 1. Fractionation of 4.0 ml rabbit sheep erythrocyte antiserum on a 2.8 × 100-cm Sephacryl S-200 column. Shaded area represents fractions which exhibited cold agglutinin activity. Fraction volumes were 4.1 ml.

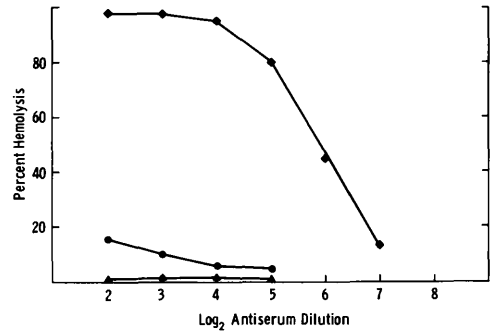

FIG. 2. Cold agglutinin activity in biphasic temperature hemolytic assay, using whole (■), Group C Streptococcal vaccine absorbed (●), or sheep erythrocyte-absorbed (▲) sheep erythrocyte antiserum.

associated with sheep erythrocyte determinants was shown by absorption experiments. Whole antiserum was absorbed once in the presence of one-half the serum volume of packed erythrocytes. In addition, the same procedure was performed using Group C streptococcal vaccine. After absorption, each solution was titrated in the biphasic temperature hemolysis assay. Shown in Fig. 2 are the results of these assays. As depicted, both absorption procedures were effective in depleting the antisera of hemolytic activity toward rabbit red blood cells. Absorption with Type III pneumococcal vaccine did not alter the original activity. Because of the apparent reactivity of the cold agglutinin material with sheep erythrocytes and Group C streptococcal vaccine, further examination involved fractionated material in ligand inhibition experiments. Cold agglutinin antibody, after fractionation by Sephacryl S-200 chromatography, was concentrated and the IgM antibody titrated to yield 50% hemolysis in the biphasic temperature hemolysis assay. This amount of antibody was subsequently preincubated in the presence of various saccharide compounds as inhibitors. Shown in Table 1 are the results of these assays. Clearly, of the polysaccharides tested, Group C streptococcal carbohydrate was the best inhibitor of hemolysis. More than 90% inhibition was effected in the presence of 100 ng carbohydrate. At a similar concentration Type III pneumococcal polysaccharide was not inhibitory. Of the individual sugars tested, *N*-acetylgalactosamine clearly yielded the most inhibition. As seen by the data in Table 1, approximately 200 μM *N*-acetylgalactosamine was sufficient to cause 50% inhibition of lysis while greater than 1200 μM melibiose was required for equivalent inhibition activity. When lactose was tested, greater than 10 mM did not cause detectable inhibition of cold agglutinin antibody: erythrocyte interaction. Thus, lactose was the least effective inhibitor. Interestingly, galactose, although significantly less reactive than *N*-acetylgalactosamine, was a more effective inhibitor than lactose (approximately 7 mM required for 50% inhibition).

Discussion. The demonstration that antibody reacts with a specific immunogen, implies antibody has arisen in direct response to the immunogen, and not indirectly as a result of nonspecific B lymphocyte activation. It is possible, however, that under hyperimmune conditions, non-antigen-specific soluble activation factors may stimulate nearby lymphocytes to elicit a primary response. Certain cold agglutinin antibody responses may fall into this category. For example, cold agglutinin antibodies which appear during infectious illness, or during experimental animal hyperimmunization can be shown to react with

TABLE I. INHIBITION CHARACTERISTICS OF VARIOUS SACCHARIDES AND POLYSACCHARIDES IN BIPHASIC TEMPERATURE HEMOLYTIC INHIBITION ASSAY

Inhibitor	Amount required for 50% inhibition	
	m*M*	ng
N-Acetylgalactosamine	0.22	—
Melibiose	1.78	—
Galactose	7.08	—
Lactose	No inhibition at 10.0	
Group C carbohydrate[a]	—	42
Type XIV carbohydrate[b]	—	119

[a,b] These materials were of heterogeneous molecular weight; therefore, weight values are provided.

both bacterially associated and autologous erythrocyte-associated structures. Partially because of the antigenic complexity of these systems, it has been particularly difficult to determine whether (1) non-antigen-specific immune stimuli, (2) bacteria or products in association with erythrocytes, or (3) erythrocyte cross-reactive bacterial determinants, have led to production of cold agglutinin antibodies. The present observation that sheep erythrocytes can, albeit rarely, lead to a cold agglutinin antibody response, suggests a cross-reactive antibody may be generated.

It is important to note that results of the present work which utilized an innocuous agent, sheep erythrocytes, as the immunogen are similar to those obtained in other investigations which utilized bacterial vaccines. As was observed in the present study, galactose-containing ligands appeared to react best with the cold agglutinin antibody. It is also important to note, however, that the cold agglutinin antibody described in the present investigation is clearly different from the antibody described earlier in association with Group C streptococcal vaccine immunization (10). First, a significantly different sensitivity to lactose inhibition is evident since lactose was not inhibitory in the present case at a concentration which inhibited 50% of the hemolytic activity of the previously described and characterized IgM antibody. In addition, these cold agglutinin antibodies did not discriminate between I or i determinants. Human adult and cord blood O-positive erythrocytes were equivalently agglutinated at 4°. Further, agglutination of these cells occurred at 37°. Since sheep erythrocytes do not carry detectable I or i determinants, these data suggest that reactivity with human and rabbit erythrocytes by these cold agglutinin antibodies does not involve specific discriminatory recognition of I or i determinants. This observation does not, however, exclude the possibility that these sugar-reactive cold agglutinin antibodies react with galactosyl moieties on the I or i oligosaccharide complex. Similar to the cold agglutinin-containing antistreptococcal sera reported in a previous investigation, the single antiserum in the present study reacted in both the warm and cold with sheep erythrocytes, but only in the cold with rabbit erythrocytes. The Forssman glycolipid glycan portion is identical to the immunodominant Group C streptococcal carbohydrate determinants. It appears, therefore, that an immune response to Forssman antigen determinants could explain the source of rabbit erythrocyte autoreactivity. Whether other antigens were involved in the present response is not known.

The cold agglutinin antibodies presently described reacted best, but not exclusively, with *N*-acetylated-α-anomers. This interpretation is based upon the data which clearly revealed preferential reactivity with *N*-acetylgalactosamine versus galactose and melibiose versus galactose and lactose. Apparently, the 100% β-anomeric structure present in the β 1$\rightarrow$4 linkage of the lactose molecule prevented significant reactivity with the antibody combining site. Further,

the fact that lactose is a disaccharide per se is not relevant to the absence of inhibition observed in the presence of this component, since melibiose was significantly inhibitory.

That cold agglutinin antibody may appear during immunization with erythrocytes in addition to immunization with various bacterial vaccines, strongly suggests the response is specific for sheep erythrocytes and is independent of bacterial components in association with autologous erythrocyte membranes. These data therefore suggest that a clearer understanding of erythrocyte autoreactive responses could be attained through careful use of defined carbohydrate immunogenic stimulation.

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